**Abstract**

SURFIN\(_{1,2}\) is a parasite-infected red blood cell (iRBC) surface associated protein of *Plasmodium falciparum*. To analyze the region responsible for the intracellular trafficking of SURFIN\(_{1,2}\) to the iRBC and Maurer’s clefts, a panel of transgenic parasite lines expressing recombinant SURFIN\(_{1,2}\) fused with green fluorescent protein was generated and evaluated for their localization. We found that the cytoplasmic region containing a tryptophan rich (WR) domain is not necessary for trafficking, whereas the transmembrane (TM) region was. Two PEXEL-like sequences were shown not to be responsible for the trafficking of SURFIN\(_{1,2}\), demonstrating that the protein is trafficked in a PEXEL-independent manner. N-terminal replacement, deletion of the cysteine-rich domain or the variable region also did not prevent the protein from localizing at the iRBC or Maurer’s clefts. A recombinant SURFIN\(_{1,2}\) protein possessing 50 amino acids upstream of the TM region, TM region itself and a part of the cytoplasmic region was shown to be trafficked into the iRBC and Maurer’s clefts, suggesting that there are no essential trafficking motifs in the SURFIN\(_{1,2}\) extracellular region. A mini-SURFIN\(_{1,2}\) protein containing WR domain was shown by Western blotting to be more abundantly detected in a Triton X-100-insoluble fraction, compared to the one without WR domain. We suggest that the cytoplasmic region containing the WR may be responsible for their difference in solubility.

**Keywords:** malaria; Maurer's clefts; *Plasmodium falciparum*; protein trafficking; SURFIN

1. Introduction

During its asexual replication in the human host, *Plasmodium falciparum*, the apicomplexan parasite responsible for malaria, dramatically remodels the infected red blood cell (iRBC) [1]. This process involves the generation of a parasitophorous vacuole (PV) in which parasites reside and replicate, the transportation of parasite proteins into the iRBC across the PV membrane (PVM), the generation of parasite-derived membranous structures in the cytoplasm of the host RBC called Maurer’s clefs that play a major role as protein-sorting points, and the formation of knobs on the iRBC surface [2-4]. Some of the most severe malaria pathologies caused by *P. falciparum*, such as cerebral and placental malaria, are specifically linked to the adherence of the iRBCs to capillary vessels (cytoadhesion) and to uninfected RBCs (rosetting). *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a parasite protein transported to the surface of the iRBC has previously been shown to mediate these phenomena [5-7]. Understanding the molecular mechanisms and pathways by which parasite-proteins such as PfEMP1, are trafficked to the cytosol and thence to the surface of the iRBC is, therefore, critical for a clear insight into the pathogenesis of *P. falciparum* malaria.

RBCs lack a protein secretory apparatus so the parasite must establish *de novo* secretion machinery within the host cell cytoplasm in order to transport its own proteins into the iRBC across the PVM. The mechanisms that enable such trafficking are incompletely understood; however, many *P. falciparum* proteins destined for export into the iRBC contain both an N-terminal hydrophobic signal sequence and a short conserved pentameric host cell-targeting motif (RxLxEx/Q/D) termed the *Plasmodium* export element (PEXEL) or the vacuolar transport signal (VTS) [8,9]. The N-terminal signal sequence is required for the proteins to enter the constitutive secretory pathway via the endoplasmic reticulum (ER) [10,11], where the PEXEL/VTS motif is cleaved by plasmepsin V, an ER residing aspartic protease [12,13], and the newly formed N-terminus (xEx/Q/D) allows translocation into the iRBC cytosol by a PVM residing translocon called the “*Plasmodium* translocon of exported proteins” (PTEX) complex [14]. Our understanding of the mechanisms behind the transport of proteins within the iRBC remains vague, but some tentative explanations have been raised; transport may be mediated through vesicles, through complex membrane networks, non-lipid enclosed protein aggregates, or lipid enclosed structures such as Maurer’s clefs [4,15].

A few hundred proteins are known to contain a PEXEL/VTS motif, defining a large *Plasmodium* exportome [9,16]. However, several parasite proteins that are transported to the iRBC lack both an N-terminal signal sequence and a PEXEL/VTS motif, and are termed “PEXEL negative exported proteins” (PNEPs) [17]. The most well-characterized PNEPs are skeleton-binding protein 1 (SBP1)
[18], the membrane associated histidine-rich protein 1 (MAHRP1) [19] and the ring-exported proteins 1 (REX1) and 2 (REX2) [20, 21, 17]. Most of the reported PNEPs are believed to be trafficked to the iRBC via the classical secretory pathway, involving initial transport to the ER, but no shared signal or transport-related sequence has been identified to date for subsequent transport to the iRBC. SBP1, MAHRP1 and REX2 lack a signal peptide but contain a transmembrane (TM) region which, along with sequences at their N-terminal region, has been implicated in protein transport [22 – 24]; however, the hydrophobic N-terminal region of the REX protein has been shown to be the only region required for transport of this particular protein. [25].

The recently identified P. falciparum surface-associated interspersed gene (surf) family encodes high molecular mass proteins, and one of these, SURFIN4,2, has been shown to be co-transported along with PEMP1 and RIFIN to the iRBC surface [26]. The N-terminal of SURFIN4,2, predicted to be extracellular, contains a moderately conserved cysteine-rich putative globular domain (CRD) preceding a variable segment (Var) followed by a putative TM region. The C-terminal region of SURFIN4,2 contains three tryptophan-rich (WR) domains which are highly conserved among SURFIN protein members, interspersed by stretches of higher variability. The protein does not appear to contain either a hydrophobic signal peptide sequence or a classical PEXEL motif. Although there are two PEXEL-like sequences located at the N-terminal segment amino acid positions (aa) 24 - 30 (R_4D_L_E) and aa 118 - 122 (R_4_L_D), they may not be true signals because the first does not completely agree with the consensus PEXEL motif, and the second was located in the putative globular domain CRD. This suggests that SURFIN4,2 may be transported via a PEXEL-independent pathway. Using a serial deletion approach, we have attempted to identify the regions involved in P. falciparum SURFIN4,2 transport into the iRBC. We generated a panel of transgenic parasite lines expressing green fluorescent protein (GFP)-tagged recombinant SURFIN4,2 and show that the protein is trafficked as a PNEP. We show that the TM region, the only predicted hydrophobic region of the protein, is necessary for entry into the parasite’s secretory pathway and for subsequent trafficking into the iRBC. These findings confirm the importance of hydrophobic regions for the trafficking of PNEPs.

2. Materials and Methods

2.1. Plasmid construction

A panel of plasmids that were used to make final P. falciparum transfection constructs were prepared based on the Multisite Gateway® system (Invitrogen, Carlsbad, CA) [27]. DNA fragments containing attB1 and attB2 sites were inserted into pUC19, resulting in the pB12 plasmid. DNA fragments encoding aa 1 – 419 of SURFIN4,2 (CRD and a part of Var), a triple HA tag, and aa 734 – 764 of SURFIN4,2 (TM) were ligated into pB12 to make the pB12-SURF4,2-CRD-Var1-HA-TM plasmid. DNA fragments encoding aa 765 - 1347, 1320 - 1728 or 1712 - 2380 of SURFIN4,2 (first, second or third WR domain) were ligated into pB12-SURF4,2-CRD-Var1-HA-TM, resulting in the pB12-SURF4,2-CRD-Var1-HA-TM-WR1, pB12-SURF4,2-CRD-Var1-HA-TM-WR2 or pB12-SURF4,2-CRD-Var1-HA-TM-WR3, respectively. DNA fragments encoding 50 amino acids at aa 684 - 734 adjacent to TM region, a triple HA tag, TM, and WR1 regions were ligated into pB12, resulting in the pB12-SURF4,2-Var-C-HA-TM-WR1. All DNA fragments were amplified from P. falciparum 3D7 line parasites using KOD Plus DNA polymerase (Toyobo). pB12-SURF4,2-CRD-Var1-TM was further modified by site-directed mutagenesis using oligonucleotides with desired modifications as follows: To abolish the PEXEL-like sequences at aa 25 - 29 (R_25_K_27_F) or aa 118 - 122 (R_118_T_120_D), these sequences were replaced to A_25_K_A_27_F_A_29 or A_118_T_A_120_A_122, respectively, yielding pB12-SURF4,2-CRD-Var1-HA-TM-Pexel-1mut or pB12-SURF4,2-CRD-Var1-HA-TM-Pexel-2mut. To assess the N-terminal sequence for the trafficking, the N-terminal region at aa 1 – 42 (M_L_F_V_VE_L_D_S_R_L_K_S_D_A_K_R_S_V_K_R_F_K_I_E_Y_V_E_D_K) of SURFIN4,2 was replaced with the N-terminal region at aa 1 - 15 (M_I_D_V_H_V_N_Q_L_K_N_S_P_D) of P. falciparum adenylosuccinate lyase (ASL, PFB0295w), a P. falciparum enzyme not considered to be transported to the iRBC, to yield pB12-SURF4,2-CRD-Var1-HA-TM-RepN. pB12-SURF4,2-Var1-HA-TM and pB12-SURF4,2-CRD-HA-TM were generated from pB12-SURF4,2-CRD-Var1-TM by removing a region encoding aa 46 - 196 containing the CRD and a region encoding aa 198 - 419 containing the Var1 region, respectively. These pB12-based plasmids were subjected to a BP recombination reaction with pDONR™-221 (Invitrogen) according to the manufacturer’s instructions, resulting in the corresponding pENT12 Gateway Entry vectors. These pENT12-based plasmids were then subjected to a Gateway MultiSite LR recombination reaction with other Entry vectors, pCRT 5'-pENTR/4/1 (as a promoter component) and GFPm2-pENTR2/3 (as a tag sequence), and a Destination vector, pCHDR-3/4 (a kind gift from Dr. G. McFadden), according to the manufacturer’s instruction [27]. Initially, we used the promoter region of SURFIN4,2, however, the signal was very weak, thus we decided to use CRT (chloroquine resistance transporter; MAL7P1.27) promoter, which has been used to study P. falciparum protein trafficking to the iRBC cytosol and Maurer’s clefts, to overexpress recombinant proteins for the visualization of their clear location. Previous transcriptome data indicated that the promoter activity of CRT was stronger than that of SURFIN4,2 and that CRT was mainly transcribed at schizont, ring and early trophozoite stages, slightly longer than SURFIN4,2, which was mainly transcribed at schizont and early ring stages. [28, 29]. Obtained plasmids were verified by their restriction enzyme digestion pattern and sequencing. Schematic structures of the recombinant proteins expressed from the episomal form of the plasmids in the transfected P. falciparum are shown in figure 1. Detailed information may be found in Supplementary material.

2.2. Parasite culture and transfection

The P. falciparum MS822 line was used in this study. This line was isolated in Mae Sot, Thailand in 1988, maintained in vitro for less than 3 months, and kept at the Institute of Tropical Medicine, Nagasaki University [30]. Parasites were cultured in RPMI-1640 medium containing 5% heat-inactivated pooled type AB human serum and 0.25% Albumax II (Invitrogen), 200 mM hypoxanthine (SIGMA, St. Louis, MO), 10 µg/mL gentamycin (Invitrogen) and human RBC (type O) at 2% hematocrit. Human RBCs and plasma were obtained from the Nagasaki Red Cross Blood Center.
Serum was produced from the acid-citrate-dextrose-containing plasma by removing the clot that had formed after adding calcium. *P. falciparum* transfection was performed essentially as previously described [31]. Briefly, RBCs were resuspended in 400 µL of incomplete Cytomix (120 mM KCl, 0.15 mM CaCl₂, 2 mM EGTA, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, and 25 mM Hepes) containing 100 µg of plasmid DNA. Electroporations were performed in 2 mm cuvette using a Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA) with a condition of 320 mΩ, 0.32 kV and 975 µF. Observed time constants were 15-30 msec. Parasites were synchronized to ring stage by 5% sorbitol treatment, then 40 hours later, mature trophozoites/schizonts-iRBCs were resuspended with the plasmids-preloaded RBCs (final 0.2% parasitemia). At day 3 post transfection, 5 nM of the anti-folate drug WR99210 (kind gift from Dr. D. Jacobus) was supplied to the culture medium, and was maintained until drug-resistant parasites reappeared. Resistant parasites were usually detected before the 30th day of culture in the presence of the drug and were subsequently maintained in culture containing 25 nM WR99210.

2.3. Fluorescence live imaging and indirect immunofluorescence assay (IFA)

For fluorescence live imaging, 10 µL of parasite culture were incubated with 1 µg/mL of Hoechst 33342 (Molecular Probe) for 30 min at 37°C and placed on a glass slide for observation. Parasites expressing GFPm2 were visualized using a fluorescence microscope (Eclipse 80i; Nikon, Japan) and a digital camera (VB-7010; Keyence, Japan) equipped with 100 × oil immersion lens. For IFA, thin smears of *P. falciparum* iRBCs were prepared on glass slides, fixed with 4% paraformaldehyde/0.075% glutaraldehyde in PBS at room temperature for 5 min, rinsed with 50 mM glycine in PBS, and blocked with PBS containing 3% BSA (SIGMA) for 30 min [32]. For single staining, the smears were reacted with rabbit anti-GFP polyclonal antibody (ab6556; Abcam, Cambridge, UK), followed by Alexa-Fluor 488-conjugated secondary anti-rabbit IgG antibody (Invitrogen). For double staining, rabbit anti-GFP antibody and rabbit anti-SBP1 antibody (a kind gift from Dr. T. Tsuboi) were labeled with Alexa-Fluor 488 and -594, respectively, using Zenon® Rabbit IgG labeling kit (Invitrogen). The smears were then incubated with rabbit anti-GFP antibody (1:500) and rabbit anti-SBP1 antibody (1:1000) in PBS containing 3% BSA for 1 hour at 37°C. Parasite nuclear staining was carried out by adding 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen; final 1 µg/mL). Stained parasites were mounted with ProLong® Gold antifade reagent (Invitrogen) and visualized as described above. Some images were analyzed by using ImageJ software (1.44p; http://rsbweb.nih.gov/ij/).

2.4. Extraction of parasite proteins and Western blot analysis

Mature trophozoite and schizonts-iRBCs were collected by centrifugation on a 40/70% Percoll-sorbitol gradient. The enriched parasite fractions (2 – 4 × 10⁸ parasites) were subjected to protein extraction during which process the water-soluble fraction was collected following a freeze-thaw procedure in PBS containing a mixture of protease inhibitors (PI; cOmplete; Roche, Basel, Switzerland) repeated three times. The pellets were washed twice with PBS-Pi, and proteins further extracted in PBS-Pi containing 1% Triton X-100 (Tx; Calbiochem) for 30 min on ice. The insoluble materials were washed twice with PBS-Pi-Tx, then proteins were further extracted by incubation with PBS-Pi.
containing 2% SDS (Nacalai, Japan) for 30 min at room temperature.

Parasite extracts were subjected to electrophoresis on 5-20% SDS-polyacrylamide gradient mini gels (ATTO, Japan) under reducing conditions. The protein bands were transferred from gels to PVDF membranes (Millipore, Billerica, MA). The membranes were then probed with rabbit anti-GFP polyclonal antibody (1:500; ab6556; Abcam), for 1 hour at room temperature followed by a secondary incubation with the horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Promega) at a concentration of 1:25,000. Purified mouse anti-glycophorin A antibody (CD235a; BD Pharmingen) was used to detect glycophorin A as a positive control. Bands were visualized with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) and detected using a chemiluminescence detection system (LAS-4000EPUVmini; Fujifilm, Japan). The relative molecular sizes of the proteins were calculated based in reference to the molecular size standards (Precision Plus Dual Color Standards; Bio-Rad).

3. Results

3.1. Successful generation of mini-SURFIN42 proteins

The production of *P. falciparum* transfectants expressing very large proteins such as SURFIN42 (predicted molecular weight of 286 kDa in 3D7 parasite line) is technically challenging. Therefore, in order to investigate which region is responsible for the trafficking of SURFIN42 into the iRBCs, and hence Maurer's clefts, we first attempted to generate and evaluate mini-SURFIN42 proteins containing regions that are conserved among SURFIN members; N-terminal 419 amino acids containing N-terminal segment (aa 1 - 50) and CRD (aa 51 - 195), which is known among *P. falciparum* SURFIN members, and the N-terminal side of the variable region (Var1; aa 196 - 419), which is relatively conserved between SURFIN42 and SURFIN41 (identity 19.5% and similarity 28.7%), TM, and either one of the WR domains (WR1, WR2, or WR3). All mini-SURFIN42 proteins, SURFIN42-CRD-Var1-TM-WR1, -WR2, or -WR3, were tagged with GFP for visualization (Fig. 1). Although the extracellular region was tagged with triple HA, anti-HA mouse monoclonal antibody (4B2; Wako, Japan) could not detect any signals by IFA and Western blot analysis with unknown reason, thus HA tag was not used in this study. Live imaging using a fluorescence microscope produced only very weak fluorescent signals, and so the precise location of the GFPm2-tagged protein was impossible to assess. By double staining IFA, all mini-SURFIN42 proteins were observed in the iRBC cytoplasm as punctuate dots that co-localized with the Maurer's cleft protein SBP1 [18], indicating that all 3 mini-SURFIN42 proteins were transported to the Maurer's clefts (Fig. 2). In addition to the Maurer's cleft localization signal, diffuse fluorescence was also observed in the parasite, as well as in the iRBC. As all of the mini-SURFIN42 proteins were able to traverse the PVM and reach the iRBC cytosol and the Maurer's clefts, we selected SURFIN42-CRD-Var1-TM-WR1 for further evaluation.

3.2. The TM region, but not the WR domain is essential for the SURFIN42 trafficking to the iRBC and Maurer's clefts

In order to evaluate the importance of the TM region and WR domain in SURFIN42 trafficking, we generated parasites expressing only SURFIN42-CRD-Var1 (TM and WR were removed) or SURFIN42-CRD-Var1-TM (WR was removed). By live imaging, weak, but detectable GFP signals were observed for these transfectants, and we found that SURFIN42-CRD-Var1 was exclusively localized in the parasite cytoplasm, as would be expected for a protein lacking a signal sequence for transport to the ER. We speculate that the protein was located within the parasite cytosol (Fig. 3A). In contrast, SURFIN42-CRD-Var1-TM was observed in a punctate dot pattern in the iRBC, in addition to diffuse fluorescence in the parasite cytoplasm. By double staining IFA, SURFIN42-CRD-Var1 was observed only in the parasite, confirming the live imaging results, whereas SURFIN42-CRD-Var1-TM colocalized with SBP1, indicating a Maurer's cleft localization (Fig. 3B). Thus, the IFA data indicate that the SURFIN42 cytoplasmic region containing WR domain is not required for trafficking to the iRBC or Maurer's clefts, but the TM region is essential.

A diffused fluorescence pattern in the iRBC, as observed for the mini-SURFIN42 proteins, appeared to be reduced for SURFIN42-CRD-Var1-TM with the double staining IFA images. Because the single staining with
Alexa-Fluor 488-conjugated secondary antibody gave clearer images than the double staining using the Zenon antibody-labeling kit. We used two representative single staining images to measure and compare the signal intensity of the recombinant proteins in the iRBC cytosol for SURFIN\textsubscript{a}CRD-Var1-TM and SURFIN\textsubscript{a}CRD-Var1-TM-WR1. After subtracting background signals, signal intensities in the iRBC cytosol for SURFIN\textsubscript{a}CRD-Var1-TM were 13 to 22 units (Fig. 4B; CRD-Var1-TM #1 and #2), whereas those for SURFIN\textsubscript{a}CRD-Var1-TM-WR1 were 66 and 69 units (Fig. 4B; CRD-Var1-TM-WR1 #1 and #2, respectively). This indicates that the fluorescence signal in the iRBC cytosol is weaker for SURFIN\textsubscript{a}CRD-Var1-TM than SURFIN\textsubscript{a}CRD-Var1-TM-WR1 and suggests that the SURFIN\textsubscript{a}CRD-Var1-TM is less abundant in iRBC cytosol than SURFIN\textsubscript{a}CRD-Var1-TM-WR1.

In order to evaluate their solubility, parasite proteins were sequentially extracted by a repeated-freeze thaw procedure (FT; water-soluble fraction protein), followed by Tx extraction (Tx; membrane bound protein), and SDS extraction (SDS; Tx-insoluble fraction) and were detected with rabbit anti-GFP antibody. About 105-kDa bands were detected for SURFIN\textsubscript{a}CRD-Var1 and SURFIN\textsubscript{a}CRD-Var1-TM and a 230-kDa band for SURFIN\textsubscript{a}CRD-Var1-TM-WR1 by Western blot. Expected band sizes were 83, 86, and 158 kDa, respectively (Fig. 5A). Although the band sizes detected by Western blot are much larger than the expected size, this is not an uncommon observation for \emph{P. falciparum}-derived proteins which have a deviated amino acid composition due to a highly A/T-rich genome (76.3% in the exon) [33]. In addition to the target protein bands, a ~60-kDa band was observed for all fractions (Fig. 5A), but this band was also observed in the extract from the wild-type non-transfected MS822 parasite, and so was not derived from the recombinant proteins expressed in the transfected parasite lines. Although the identity of this band is unclear, it is likely derived from parasites, because this band was not observed in the extract from the parasite-uninfected RBC (Fig. 5B). Positive glycochrome A bands for the extracts from both the wild-type parasite and the uninfected RBC indicated the protein extraction from the uninfected RBC was successful. It should be noted that the rabbit anti-GFP antibodies did not show any signal when wild-type parasites were subjected to IFA. We found that SURFIN\textsubscript{a}CRD-Var1 was exclusively detected in the soluble FT fraction, indicating that this protein was in soluble form, which is consistent with the observation of its localization in the parasite's cytoplasm (Fig. 3). SURFIN\textsubscript{a}CRD-Var1-TM was detected in the Tx-soluble fraction more abundantly than in the Tx-insoluble SDS fraction. Conversely, SURFIN\textsubscript{a}CRD-Var1-TM-WR1 was detected in the SDS fraction more abundantly than in the Tx fraction. Thus both proteins appeared to be associated with membrane structures, and the cytoplasmic region containing the WR1 may be responsible for their difference in solubility.

3.3. SURFIN\textsubscript{a}\textsubscript{42} is trafficked to the iRBC cytosol in a PEXEL-independent manner

SURFIN\textsubscript{a} contains two PEXEL-like sequences, one was termed Pexel 1 in this study and was located at the N-terminal region, spanning aa 25 - 29 (R\textsubscript{44}L\textsubscript{34}E), for which the 3rd position was isoleucine instead of leucine in the authentic PEXEL motif. The other was termed Pexel 2, and was located in the CRD at aa 118 - 122 (R\textsubscript{44}L\textsubscript{34}D). In order to evaluate their involvement in the transport of the protein into the iRBC, we generated two parasite lines expressing SURF\textsubscript{a}2CRD-Var1-TM-Pexel-1mut or SURF\textsubscript{a}2CRD-Var1-TM-Pexel-2mut, for which the conserved residues of the PEXEL-like sequence were replaced by alanine (A\textsubscript{44}A\textsubscript{34}A or A\textsubscript{118}A\textsubscript{122}A, respectively). Double staining IFA revealed that SURFIN\textsubscript{a}2CRD-Var1-TM-Pexel-1mut and -2mut both showed a punctate dot pattern in the iRBC that colocalized with the Maurer's cleft protein SPB1 along with parasite localized fluorescence (Fig. 6A). Thus, there was no appreciable difference between the trafficking of these proteins and that of the original SURFIN\textsubscript{a}2CRD-Var1-TM recombinant protein. These observations suggest that the PEXEL-like sequences of SURFIN\textsubscript{a}2 play no evident function in the transport of SURFIN\textsubscript{a}2 to the iRBC cytosol.
and Maurer's clefts. Thus, SURFIN$\alpha_{4}$ is being trafficked as a PNEP.

3.4. Removal of N-terminal 42 amino acid segment, CRD, or Var1 did not prevent the SURFIN$\alpha_{4}$ trafficking to the iRBC cytosol and Maurer's clefts.

To further evaluate the importance of the different regions of the SURFIN$\alpha_{4}$ extracellular region in the trafficking of the protein to the iRBC, we generated three following parasite lines: Two lines expressing SURFIN$\alpha_{4}$Var1-TM or SURFIN$\alpha_{4}$CRD-TM, in which the CRD or Var1 region were deleted from SURFIN$\alpha_{4}$CRD-Var1-TM and the one line expressing SURFIN$\alpha_{4}$CtVar1-TM-RepN, in which the N-terminal first 42 amino acids of SURFIN$\alpha_{4}$CRD-Var1-TM was replaced by the N-terminal first 15 amino acids of *P. falciparum* adenylsucinate lyase (PfASL), an enzyme involved in the purine metabolism in the cell cytosol and is not considered to be transported to the iRBC [34]. Double staining IFA revealed that SURFIN$\alpha_{4}$Var1-TM and SURFIN$\alpha_{4}$CRD-TM colocalized with SBP1 in a punctate dot pattern in the iRBC and was also present in the parasite cytoplasm. No difference was observed between these two lines and the line expressing SURFIN$\alpha_{4}$CRD-Var1-TM (Fig. 6B). A more diffused iRBC localization with less obvious dot pattern formation was observed with the line expressing SURF1$\alpha_{4}$CtVar1-TM-RepN compared to that expressing SURFIN$\alpha_{4}$CRD-Var1-TM. Nonetheless, the transport of the SURFIN$\alpha_{4}$CRD-Var1-TM-RepN protein was not completely abrogated and signals, although faint, still colocalized with the Maurer's cleft SBP1. Thus, any of the N-terminal segment (aa 1 - 42), the CRD (aa 46 - 196), or the variable region (aa 198 - 733) in the extracellular region of SURFIN$\alpha_{4}$ do not carry a specific motif necessary for protein transport to the iRBC.

To confirm these findings, we truncated the entire external domain from the mini-SURFIN$\alpha_{4}$ protein, and added 50 amino acids (SSGQVRRSGGGQGETYIVGTSQGFHKEVIPSISKDGK SGKTQIVSNK) preceding the TM region in order to support the integrity of the TM region for membrane insertion, to generate a parasite expressing SURFIN$\alpha_{4}$Var-C-TM-WR1, thus this protein contains 50 amino acids derived from SURFIN$\alpha_{4}$ followed by a triple HA tag as an extracellular region. The recombinant protein was transported to the iRBC and observed in a punctuate dot pattern in the iRBC cytosol, colocalizing with SBP1 (Fig. 7). This indicates that the extracellular region of SURFIN$\alpha_{4}$ is not required for the trafficking of the protein to the iRBC.

4. Discussion

In this study, we generated GFPm2-fused mini-SURFIN$\alpha_{4}$ proteins that, following their transfection into a *P. falciparum* parasite line, was observed to be trafficked into the iRBC and Maurer's clefts. Using this system, we then attempted to identify the specific region of the protein responsible for the iRBC and/or Maurer's cleft localization. We found that the TM region, but not the cytoplasmic region containing WR domain was essential for protein transport. We consider it likely that the TM region is responsible for initiating the trafficking of the protein into the ER. Two PEXEL-like sequences were found not to be essential for the movement of the protein into the iRBC and Maurer's clefts, indicating that SURFIN$\alpha_{4}$ trafficking is PEXEL-independent. N-terminal replacement, deletion of the CRD or Var region did not prevent iRBC and Maurer's cleft localization, suggesting that no trafficking motif exists in these regions.

By sequential extraction of recombinant SURFIN$\alpha_{4}$ proteins, we found that mini-SURFIN$\alpha_{4}$ with an intact WR domain showed more resistance to Triton-X 100 extraction than a similar protein in which WR domain had been removed. As endogenous SURFIN$\alpha_{4}$ was insoluble in Triton-X 100 but soluble in SDS [26], we suggest that the cytoplasmic region, probably the WR domain, contributes to this difference. Insolubility with a neutral detergent such as Triton-X 100, was also reported for PfEMP1 [35]. A large degree of sequence similarity was shown between the cytoplasmic WR domain of SURFIN$\alpha_{4}$, PfEMP1, and another iRBC protein P332 [26]. The cytoplasmic regions of both PfEMP1 and P332 are known to bind to RBC actin, the former also binding to spectrin [36, 37]. Therefore, we suggest that the WR domain of SURFIN$\alpha_{4}$ also associates with the RBC cytoskeleton, although further evaluation is required to assess this hypothesis.

Similar to most of the PNEPs reported so far, the SURFIN$\alpha_{4}$ TM region was found to be essential for protein trafficking. PfSBP1 [11], MAHRP1 [19] and REX2 [24] share this feature, with their TM regions known to play
important roles in protein transport. However, the 
N-terminal sequence of these PNEPs was also found to be 
additional to correct protein trafficking. In addition 
to the TM region, P/SBP1 was shown to require the N-terminal 
segment at aa 16 – 26, which contain highly negative net 
charge residues (DEPTQLQDVAP) for transport into the 
irBC [11]. This may also be the case for MAHRP1, as the 
N-terminal 50 amino acids of this protein, which is acidic, 
along with P/SBP1 TM region is able to transport protein 
to the irBC [11]. Conversely, REX2 appears to contain 
region resembling a PEXEL motif after cleavage in the ER 
at aa 5 – 10 (LxExE,hhS,y; h indicates hydrophobic residues), 
for which only the glutamate residue at aa 7 was found to be 
critical for trafficking [24]. In the mini-SURFN1,2 proteins 
we expressed in this study, none of the regions from the 
SURFN1,2 extracellular regions shown to be indispensable 
for trafficking to the irBC, thus the trafficking of 
SURFN1,2 appears not depend on specific sorting signals, 
nor potential escorter proteins, but other factors in addition 
to the TM region.

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References
[1] Tilley L, Sougrat R, Lithgow T, Hanssen E. The twists and 
C. Maurer’s clefts: a novel multi-functional organelle in the 
Tilley L, Cowman AF. Trafficking and assembly of the 
[5] Baruch DJ. Adhesive receptors on malaria-parasitized red 
[6] Chen Q, Schlichterle M, Wahlgren M. Molecular aspects of 
Fagan T. Cytoadherence, pathogenesis and the infected red 
[8] Hilleter NL, Bhattacharjee S, van Ooj C, Lioiios K, Harrison T, 
Lopez-Estraño C, Haldar K. A host-targeting signal in 
Targeting malaria virulence and remodeling proteins to the 
P/SBP1 to the Plasmodium falciparum Maurer’s clefs. 
[12] Boddey JA, Hodder AN, Günther S, Gilson PR, Patsiouras H, 
Kapp ÉA, et al. An aspartyl protease directs malaria effector 
A, Goldberg DE. Plasmpesin V licenses Plasmodium proteins 
Penfuss AT, et al. A newly discovered protein export 
Vigan-Womas I, et al. Identification of a role for the 
P/EMP1 semi-conserved head structure in protein trafficking to the 
surface of Plasmodium falciparum infected red blood cells. 
[16] van Ooj C, Tamez P, Bhattacharjee S, Hiller NL, Harrison T, 
Lioiios K, et al. The malaria secretome: from algorithms to 
essential function in blood stage infection. PLoS Pathog 2008; 
4:e1000084.
[17] Spielmann T, Hawthorne PL, Dixon MW, Hannemann M, 
Klotz K, Kemp DJ, et al. A cluster of ring stage–speciﬁc genes 
linked to a locus implicated in cytoadherence in Plasmodium falciparum codes for PEXEL-negative and PEXEL-positive 
proteins exported into the host cell. Mol Biol Cell 2006; 
17:3613–24.
[18] Blisnick T, Morales Betoulle ME, Barale JC, Uzereau P, Berry 
L, Desroses S, et al. PfSBP1, a Maurer’s cleft Plasmodium falciparum protein, is associated with the erythrocyte skeleton. 
L, Beck HP. MAHRP-1, a novel Plasmodium falciparum 
histidine-rich protein, binds ferritroporphyrin IX and 
localizes to the Maurer’s cleft organelles. Mol Microbiol 2008; 
69:938–53.
[20] Saridaki T, Sánchez CP, Pfäpler J, Lanzer M. A conditional 
export system provides new insights into protein export in 
Plasmodium falciparum-infected erythrocytes. Cell Microbiol 
[21] Spielmann T, Gilberger TW. Protein export in malaria 
parasites: do multiple export motifs add up to multiple export 
[22] Hanse E, Heimann S, Grüning C, Heiber A, Jansen PW, 
Langer C, et al. Sequence requirements for the export of the 
Plasmodium falciparum Maurer’s clefts protein REX2. Mol 
[23] Dixon MW, Hawthorne PL, Spielmann T, Anderson KL, 
Trenholme KR, Gardner DL. Targeting of the ring exported 
protein 1 to the Maurer’s clefts is mediated by a two-phase 
Alexandre M, Saridaki T, Fröhlich KS, Braun-Breton C, Lanzer M. Targeted mutagenesis of the 
EMP1, a novel multi-functional protein in the 
6:1003–17.


